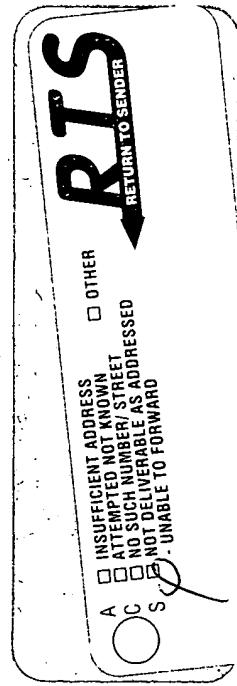
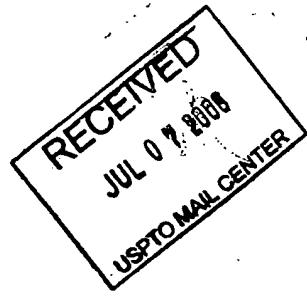


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1645
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APPLICATION NO.	RECEIVED DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/674,368	10/27/2000	Thomas N. Metcalf III	33377-00	6981

7590 06/21/2006
Alan M Gordon
American Home Products Corporation
Patent Law Department
One Campus Drive
Parsippany, NJ 07054

EXAMINER	
GANGLE, BRIAN J	
ART UNIT	PAPER NUMBER
1645	

DATE MAILED: 06/21/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/674,368	METCALF III ET AL.	
	Examiner	Art Unit	
	Brian J. Gangle	1645	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) Responsive to communication(s) filed on 27 October 2000.
- 2a) This action is FINAL. 2b) This action is non-final.
- 3) Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) Claim(s) 1-42 is/are pending in the application.
 - 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) Claim(s) _____ is/are allowed.
- 6) Claim(s) _____ is/are rejected.
- 7) Claim(s) _____ is/are objected to.
- 8) Claim(s) 1-42 are subject to restriction and/or election requirement.

Application Papers

- 9) The specification is objected to by the Examiner.
- 10) The drawing(s) filed on _____ is/are: a) accepted or b) objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
 - a) All b) Some * c) None of:
 1. Certified copies of the priority documents have been received.
 2. Certified copies of the priority documents have been received in Application No. _____.
 3. Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) Notice of References Cited (PTO-892)
- 2) Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date _____.
- 4) Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____.
- 5) Notice of Informal Patent Application (PTO-152)
- 6) Other: _____.

DETAILED ACTION

Election/Restrictions

Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I, claim(s) 1-2 and 7-8, drawn to vaccine compositions comprising a *Neisseria gonorrhoeae* pilin protein.

Group II, claim(s) 1, 3-4, and 7-8, drawn to vaccine compositions comprising a *Neisseria meningitidis* class I pilin protein.

Group III, claim(s) 1, 3, 5, and 7-8, drawn to vaccine compositions comprising a *Neisseria meningitidis* class II pilin protein.

Group IV, claim(s) 6 and 27, drawn to vaccine compositions comprising a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:2.

Group V, claim(s) 9, drawn to a method of immunizing against *Neisseria gonorrhoeae* using a vaccine composition comprising a *Neisseria gonorrhoeae* pilin protein.

Group VI, claim(s) 10, drawn to a method of immunizing against *Neisseria gonorrhoeae* using a vaccine composition comprising a *Neisseria meningitidis* class I pilin protein.

Group VII, claim(s) 11, drawn to a method of immunizing against *Neisseria gonorrhoeae* using a vaccine composition comprising a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:2.

Group VIII, claim(s) 12, drawn to a method of immunizing against *Neisseria meningitidis* using a vaccine composition comprising a *Neisseria meningitidis* class I pilin protein.

Group IX, claim(s) 13, drawn to a method of immunizing against *Neisseria meningitidis* using a vaccine composition comprising a *Neisseria gonorrhoeae* pilin protein.

Art Unit: 1645

Group X, claim(s) 14, drawn to a method of immunizing against *Neisseria meningitidis* using a vaccine composition comprising a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:2.

Group XI, claim(s) 15, drawn to a method of preparing a vaccine comprising a *Neisseria* pilin protein.

Group XII, claim(s) 16-26, drawn to DNA encoding a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:2, plasmids comprising said DNA, host cells comprising said plasmid, and a method of producing said protein by culturing said host cell.

Group XIII, claim(s) 28 and 42, drawn to vaccine compositions comprising a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:4.

Group XIV, claim(s) 29, drawn to a method of immunizing against *Neisseria gonorrhoeae* using a vaccine composition comprising a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:4.

Group XV, claim(s) 30, drawn to a method of immunizing against *Neisseria meningitidis* using a vaccine composition comprising a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:4.

Group XVI, claim(s) 31-41, drawn to DNA encoding a chimeric protein of a *Neisseria gonorrhoeae* pilin protein and SEQ ID NO:4, plasmids comprising said DNA, host cells comprising said plasmid, and a method of producing said protein by culturing said host cell.

The inventions listed as Groups I-XVI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-XVI appears to be a vaccine composition comprising an isolated and purified recombinantly expressed pilin protein of the genus *Neisseria*.

However, Rothbard *et al.* (J. Exp. Med., 160:208-221, 1984) disclose an immunogenic composition containing *Neisseria gonorrhoeae* pili (see page 209, paragraph 3). The products of the prior art reference appear to be the same as the product claimed by the applicant because they appear to possess the same or similar functional characteristics, i.e. an immunogenic composition and a vaccine comprising *Neisseria gonorrhoeae* pilin proteins. The purification or production of a product by a particular process does not impart novelty or unobviousness to a product when the same product is taught by the prior art. This is particularly true when properties of the product are not changed by the process in an unexpected manner. See In re Thorpe, 227 USPO 964 (CAFC 1985); In re Marosi, 218 USPO 289, 29222-293 (CAFC 1983); In re Brown, 173 USPO 685 (CCPA 1972). Therefore, Rothbard *et al.* meets the limitations of claim 1.

Therefore, the technical feature linking the inventions of groups I-XVI does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the art.

The examiner has required restriction between product and process claims. Where applicant elects claims directed to the product, and the product claims are subsequently found allowable, withdrawn process claims that depend from or otherwise require all the limitations of the allowable product claim will be considered for rejoinder. All claims directed a nonelected process invention must require all the limitations of an allowable product claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product are found allowable, an otherwise proper restriction requirement between product claims and process claims may be maintained.

Withdrawn process claims that are not commensurate in scope with an allowable product claim will not be rejoined. See MPEP § 821.04(b). Additionally, in order to retain the right to rejoinder in accordance with the above policy, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder.** Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species or invention to be examined even though the requirement be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention or species may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and

specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse.

Should applicant traverse on the ground that the inventions or species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions or species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C.103(a) of the other invention.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brian J. Gangle whose telephone number is (571) 272-1181. The examiner can normally be reached on M-F 8-4:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lynette Smith can be reached on (571) 272-0864. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Brian Gangle
6/8/2006



ROBERT ZEMAN
PATENT EXAMINER

Notice of References Cited		Application/Control No.	Applicant(s)/Patent Under Reexamination	
		09/674,368	METCALF III ET AL.	
Examiner		Art Unit		Page 1 of 1
Brian J. Gangle		1645		

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
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	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Rothbard et al. (J. Exp. Med., 160:208-221, 1984)
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

STRAIN-SPECIFIC AND COMMON EPITOPES OF GONOCOCCAL PILI

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Departments of Medicine and Medical Microbiology, Stanford University School of Medicine,
Stanford, California 94305*

Pili are polymeric filaments extending from the cell envelope of *Neisseria gonorrhoeae* that promote infectivity by binding to epithelial cell surfaces (1-6). Since antisera directed at pili may block mucosal adherence by gonococci and therefore their capacity to colonize urogenital epithelium, pili have been proposed as vaccine constituents for the prevention of gonorrhea (1). However, gonococcal pili are antigenically heterogeneous and the protection conferred by a vaccine composed of undenatured pilus filaments from a single gonococcal strain appears to be strain specific. Yet, antisera against pili from one strain can cross-react weakly with pili from other strains, implying that a common determinant could exist (1). Characterization of a common determinant might lead to the development of a suitable immunogen for the prevention of gonorrhea.

To investigate the structural basis of these serologic properties, we recently have determined the complete amino acid sequence of pilin isolated from the transparent colonial variant (Tr)¹ of strain MS11 and the partial sequence of R10 (Tr) pilin (7). The comparison of these two sequences and the previously reported serological properties of antisera directed against the two principal cyanogen bromide-generated fragments of pilin (CNBr-2, residues 9-92 and CNBr-3, residues 93-159) have enabled us to construct an antigenic model of the protein (8). The immunodominant, antigenically variable region is within CNBr-3, while CNBr-2 contains a conserved receptor binding region and possibly an immunorecessive epitope common to all gonococcal pilin (8).

In this study, peptides corresponding to both variable and constant regions of MS11 pilin were synthesized in order to (a) more precisely determine the antigenic structure of pilin, (b) understand the molecular details of the antigenic variation in the protein, and (c) identify regions of the protein that might elicit antisera that would inhibit the binding of all gonococcal strains to eucaryotic cells. Each peptide was tested for its ability to be bound by polyclonal sera

This work was supported, in part, by a grant from the Veterans Administration and by a grant from the Institut Merieux. G. K. S. is a Fellow of the John A. Hartford Foundation. Correspondence should be addressed to him at the Department of Medicine, Division of Infectious Diseases, Stanford University Medical School, Stanford, CA 94305.

¹ Abbreviations used in this paper: BSA, bovine serum albumin; CNBr-2, cyanogen bromide-generated fragment of pilin, residues 9-92; CNBr-3, CNBr-generated fragment of pilin, residues 93-159; DCC, dicyclohexylcarbodiimide; SMCC, succinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate; Tr, transparent colonial variant.

directed against pili isolated from homologous and heterologous gonococcal strains. In addition, antisera directed against CNBr-2 and CNBr-3 were tested for their ability to bind the peptides. An antigenic determinant common to all strains of pili and two separate strain-specific epitopes were defined. We also have demonstrated that antisera against CNBr-2 recognizes a different common determinant than that seen by sera against the intact pilus filaments.

Materials and Methods

Purification of Pili and the Generation of Cyanogen Bromide Fragments. Pili purification and the generation and isolation of the cyanogen bromide-generated fragments, CNBr-2 and CNBr-3, from MS11(Tr) and R10(Tr) strains of *N. gonorrhoeae* were performed as described previously (7).

Specific Anti-Pili Antisera. Antisera against undenatured pili from MS11(Tr), R10(Tr), and against their two major cyanogen bromide-generated fragments (CNBr-2 and CNBr-3) were prepared in female New Zealand White rabbits. 50 μ g of each protein was emulsified in 1 ml of phosphate-buffered saline with an equal volume of complete Freund's adjuvant and administered by multiple subcutaneous injections. Booster injections in Freund's incomplete adjuvant were administered 6 wk later. 10 d thereafter, the animals were bled by cardiac puncture and the sera were filter sterilized and stored at 4°C.

Selection of the Peptides Synthesized. Peptides believed to contain strain-specific epitopes were chosen according to differences in sequence between MS11 and R10 pilin. All known sequence differences between these two proteins occur between residues 98 and the carboxyl terminus, residue 159 (7, 8). Particular importance was given to regions where amino acid substitutions significantly affected either the charge, the hydrophobicity/hydrophilicity, or the predicted secondary structure of the region. Regions of sequence homology between residues 8 and 92 were synthesized in an attempt to define a common epitope. We avoided regions that were very hydrophobic, but did not simply choose areas of great hydrophilicity. The association of the hydrophilicity of a region with antigenicity neglects the importance of hydrophobic interactions when an antibody binds an antigen (9). Consequently, regions that had both hydrophobic and hydrophilic character were preferred.

The possible conformations that the peptide can adopt when conjugated to a carrier protein also were considered. Only if a peptide adopts the identical secondary structure that it has in the intact protein will it both bind sera generated against the intact protein and elicit antibodies that cross-react with that protein. Of the three common secondary structures present in proteins, β -pleated sheets, α -helices, and reverse or β -turns, we believe reverse turns have the highest probability of being present in an isolated oligopeptide <15 residues in length. Therefore, a peptide containing a region with high turn probability will be more likely to be identical to the corresponding region in the intact protein than regions predicted to be sheets or helices.

The β -turns were emphasized further by conjugating the peptide in an orientation that resulted in the β -turn being distal from the linkage to the carrier protein.

Synthesis and Characterization of Peptides. Peptides were synthesized by solid phase techniques (10) on a Beckman model 990B peptide synthesizer using commercially available amino acid polystyrene resins and t-Boc-protected amino acids (Peninsula Laboratories, Belmont, CA), with the following side chain protecting groups: O-benzyl esters for Asp, Glu, Thr, and Ser; tosyl for Arg and His; para-methoxybenzyl for Cys, ortho-chlorobenzylxycarbonyl for Lys, and 2,6-dichlorobenzyl for Tyr. All couplings were performed using a 2.5 molar excess of t-Boc amino acid and dicyclohexylcarbodiimide (DCC) over the number of milliequivalents of amino acid on the resin. In the case of Asn and Gln, a 2.5 molar excess of the amino acid, DCC, and N-hydroxy triazole was used.

All couplings were >99% complete, as determined by the reaction of the resin with ninhydrin (11). The peptides were deprotected and removed from the resin simultaneously by treatment with anhydrous hydrogen fluoride in the presence of anisole, dimethyl sulfide, and indole. The peptides were separated from the various organic side products

by extraction with ether and isolated from the resin by extraction with 5% acetic acid and subsequent lyophilization. The purity of the crude product was determined by high pressure liquid chromatography on a C-18 reverse phase column (Merck, Darmstadt, Germany) and by amino acid analysis. The peptides used in these experiments were not further purified since they all contained >90% of the desired product.

Conjugation of the Peptides to Carrier Proteins. The peptides were conjugated to bovine serum albumin (BSA) using succinimidyl 4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate (SMCC) (Pierce, Rockford, IL) as described (12). Briefly, 10 mg of the carrier protein were dissolved in 2 ml phosphate-buffered saline, pH 7.4, and combined with 5 mg of crosslinker dissolved in 500 μ l of dimethylformamide. After an hour, the conjugate was separated from unreacted crosslinker by gel filtration on a G-25 column in 0.1 M phosphate, pH 6.0. The peptide to be conjugated was dissolved in 0.1 M borate, pH 9.1 and reduced with NaBH₄ (100 μ l of 0.1 M stock). After 5 min the pH of the borate solution was lowered to 1 with 1 M HCl, to remove the excess NaBH₄, and then raised to pH 6 with 1 M NaOH and combined with the carrier-crosslinker conjugate. After incubating for an additional hour the carrier-peptide conjugate was desalted on a G-25 column in 0.1 M NH₄HCO₃. The degree of conjugation was quantitated by comparing the amino acid composition of the carrier protein before and after reaction with the peptide. The conjugates used in this study all contained between 15 and 25 peptides per molecule of BSA.

Determination of the Cross-reactivity of Sera Against Intact Pili and CNBr-2 and CNBr-3 of Pilin. A two-step competitive ELISA was used to determine the cross-reactivity of sera directed against MS11 and R10 pili and their cyanogen bromide-generated fragments, CNBr-2 and CNBr-3 (13). Briefly, each antisera was diluted to a concentration that resulted in 60% maximal binding to the antigen on a solid phase. The sera (100 μ l) containing increasing concentrations of either MS11 or R10 pili were added to wells precoated with 0.1 μ g of either MS11 or R10 pili. The plates were incubated for 3 h at 37°C, washed, treated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA), washed, treated with *p*-nitrophenyl phosphate (Sigma, St. Louis, MO), and read at 405 nm.

Binding of Polyclonal Sera to Peptide-Protein Conjugates. Rabbit polyclonal antibodies against either whole pili or cyanogen bromide-generated fragments of pilin, CNBr-2 and CNBr-3, were assayed for their ability to bind peptide-BSA conjugates by using solid phase binding assays. Briefly, 96-well plates were coated with 10 μ g of the peptide-BSA conjugate, washed, treated with serially diluted antisera, washed, and treated either with ¹²⁵I protein A (Amersham, Arlington Heights, IL) or a goat anti-rabbit alkaline phosphatase conjugate (Cappel, West Chester, PA) followed by *p*-nitrophenyl phosphate (Sigma, St. Louis, MO). The radioactive wells were cut from the plate and counted, while the wells containing the antibody-enzyme conjugates were read at 405 nm.

Absorption of Antisera with Peptide-Carrier-Sepharose Conjugates. The peptide-BSA conjugates were reacted with cyanogen bromide-activated Sepharose (Pharmacia, Piscataway, NJ) as described (14). Anti-R10 and anti-MS11 pilus sera (1 ml) were absorbed with 100 μ l of either peptide 121-134-BSA or 135-151-BSA sepharose for 2 h at room temperature. The mixture was spun and the absorbed sera was separated and assayed for the complete removal of the specific anti-peptide antibody by a solid phase binding assay.

Results

Shared Antigenicity of R10 and MS11 Pili. Previous studies by Brinton et al. (1) demonstrated that antisera against gonococcal pili are directed principally at strain-specific epitopes. However, the sera also contain antibodies that cross-react with pili of other strains. This cross-reactivity could be due to either differential binding at strain-specific epitopes as a result of amino acid sequence variations or due to the presence of a weakly immunogenic determinant common to all gonococcal pili. Polyclonal sera against the R10 and MS11 pili used in this study

initially were tested using a two-step inhibition solid phase binding assay to determine their specificity (13). As seen in Fig. 1, A and B, the anti-whole pili sera is quite strain specific. The shared antigenicity detected by both sera is only ~5%. Whether the cross-reactivity of the sera is due to epitopes common to all pili or due to differential binding at strain-specific determinants was partly answered by examining the antigenic relatedness detected by the sera against two cyanogen bromide-generated fragments of pilin, CNBr-2, residues 8–92 and CNBr-3, residues 93–159. A marked difference is apparent in the shared antigenicity detected by the sera directed against CNBr-2 (Fig. 1, C and D) from that against CNBr-3 (Fig. 1, E and F). In the former case the cross-reactivity was almost complete, whereas antisera against the carboxyl terminal fragment displayed high strain specificity. These experiments indicate that a weakly immu-

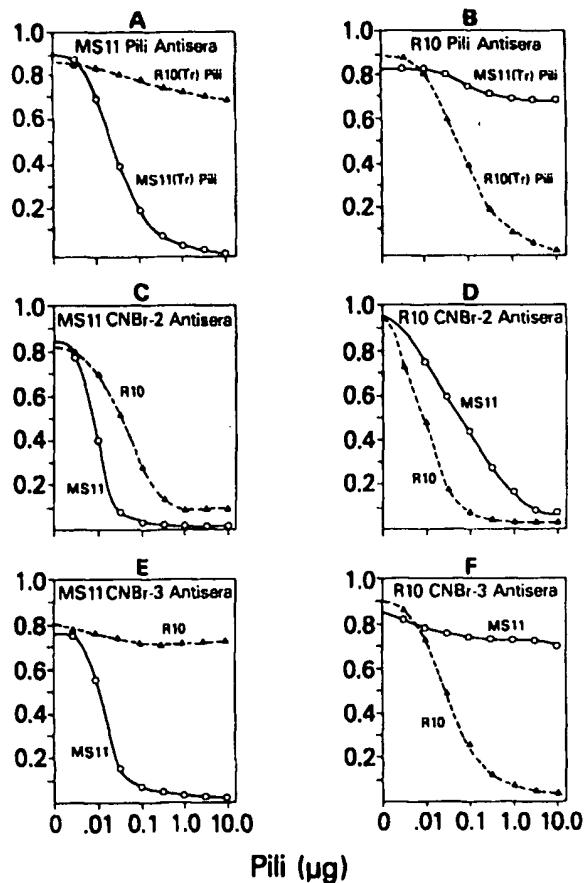


FIGURE 1. The specificity of antisera generated against either intact MS11 pili, intact R10 pili, or each of their principal cyanogen bromide-generated fragments, CNBr-2 (residues 9–92) and CNBr-3 (residues 93–159). Antisera against intact MS11 pili (A), MS11 (CNBr-2 (C), and MS11 CNBr-3 (E) were assayed in a two-step competitive ELISA for their ability to bind wells coated with MS11 (Tr) pili in the presence of increasing amounts of either soluble R10 or MS11 pili (abscissa). Similarly, antisera against intact R10 pili (B), R10 CNBr-2 (D), and R10 CNBr-3 (F) were tested for their ability to bind wells coated with R10 (Tr) pili in the presence of increasing concentrations of soluble MS11 and R10 pili (abscissa).

nogenic, common epitope(s) exists between residues 9–92 and that the strain-specific, immunodominant determinant(s) is located between residues 93–159. The precise location of each of these epitopes was determined by examining the antigenicity of synthetic peptides corresponding to regions of the MS11 sequence.

Location of Strain-specific Antigenic Determinants. To locate more precisely the strain-specific epitopes within the carboxyl terminal cyanogen bromide-generated fragment of gonococcal pilin, four peptides were synthesized corresponding to residues 95–107, 107–121, 121–134, and 135–151 (Table I). Peptides 121–134 and 135–151 compose a disulfide loop, while 95–107 and 107–121 correspond to the region on the amino terminal side of this loop. The reduced sulphydryl of the cysteine at either the carboxyl or amino terminus of each peptide was used to conjugate it to BSA using the hetero-bifunctional crosslinker SMCC. Microtiter plates were coated with the BSA-peptide conjugates and antisera against MS11 and R10 pili were tested for their ability to bind to them.

The homologous anti-MS11 serum binds the two peptides corresponding to the disulfide loop, but not the peptides outside this region (Fig. 2A). Anti-R10 serum does not bind all four of these conjugates (Fig. 2C). Additional proof that these peptides contain an epitope specific for MS11 pili is provided by binding experiments with antisera against the carboxyl terminal cyanogen bromide-generated fragments (CNBr-3) or MS11 and R10. Anti-MS11 CNBr-3 serum

TABLE I
Amino Acid Sequences of the Eight Synthetic Oligopeptides and Their Corresponding Location in MS11 Pilin

	L	P	A	Y	Q	D	Y	T	A	R	A	Q	V	S	E	G	C*
21																	35
41																	50
48																	60
69																	84
95																	
																	107
107																	
																	121
121																	
																	134
135																	
																	151

* Underlined residues do not exist in the primary structure of MS11 pilin. Additional glycine and cysteine residues were added as spacers and as a mode of attachment to carrier molecules, respectively.

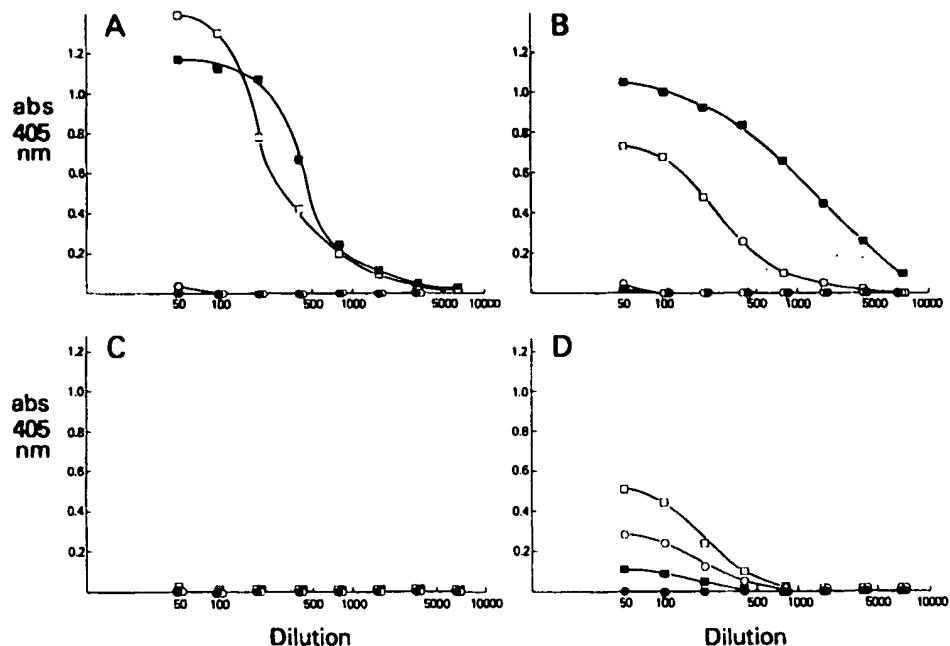


FIGURE 2. Reaction of anti-gonococcal pili sera with peptide-BSA conjugates corresponding to regions of sequence variation between strains. Anti-MS11 pili (A), anti-MS11 CNBr-3 (B), anti-R10 pili (C), and anti-R10 CNBr-3 (D) antisera were assayed for their ability to bind peptides corresponding to residues 95–107 (●), 107–121 (○), 121–134 (■), and 135–151 (□) of the MS11 pilin sequence. The enzyme-linked immuno-binding assays were performed as described in Materials and Methods. Each point represents the average of three determinations.

bound the two peptides comprising the disulfide loop even at dilutions as high as 1:6,400 (Fig. 2B), whereas antiserum to the same fragment from R10 pilin binds very weakly and only at high serum concentrations (Fig. 2D).

The observation that the two peptides that are bound both by polyclonal sera against whole MS11 pili and against CNBr-3 are juxtaposed in sequence, creates the possibility that either there is a single epitope whose component amino acids are shared by the two peptides or that there are two independent epitopes, one within each peptide. This ambiguity was resolved easily by absorbing the MS11 pilus antiserum with one peptide conjugate and then studying the absorbed serum's ability to bind the second peptide conjugate. If there is a single shared determinant, then the absorption should reduce the amount of antibodies binding to the second peptide. If there are two epitopes, the absorption should not affect the serum's ability to bind the other conjugate. Absorption with peptide 121–134 has no effect on the serum's ability to bind 135–151 and reciprocally, absorption with peptide 135–151 does not affect the binding to 121–134 (Table II). Each peptide contains a strain-specific epitope.

Identification of a Common Determinant in Gonococcal Pili. Even though the immunodominant epitopes of gonococcal pilin are strain specific, a common determinant(s) also has been demonstrated (Fig. 1) to exist within CNBr-2. Peptides chosen to span most of this region of conserved sequence between MS11 and R10 pilin were synthesized as possible epitopes (Table I). Glycine and

TABLE II
*Reaction of Anti-MS11 Pili Sera with Peptides Corresponding to Residues 121-134 and 135-151 Before and After Absorption**

Dilution	Polyclonal sera absorbed with Sepharose			Polyclonal sera absorbed with 121-134-BSA-Sepharose			Polyclonal sera absorbed with 135-151-BSA Sepharose		
	121-134-BSA	135-151-BSA	BSA	121-134-BSA	135-151-BSA	BSA	121-134-BSA	135-151-BSA	BSA
	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm
1:100	9,483	10,801	428	507	8,769	428	9,408	563	549
1:500	2,229	2,856	145	193	2,062	103	2,352	186	135
1:1,000	1,245	1,450	89	150	1,101	78	1,496	110	75
1:5,000	234	266	47	99	231	45	321	61	64
1:10,000	152	249	37	69	155	26	167	78	88

* Details of the absorption are described in Materials and Methods. Wells were coated with 10 $\mu\text{g}/\text{ml}$ of either 121-134-BSA, 135-151-BSA, or BSA, treated with either absorbed or unabsorbed sera, washed, and then exposed to 50,000 cpm of ^{125}I protein A. The values reported are averages of three separate determinations.

cysteine were added to the carboxyl terminus of each peptide to allow them to be conjugated to BSA as previously described. Antisera against both MS11 and R10 pili and against CNBr-2 of each pilin were tested for their ability to bind these conjugates. Anti-whole pili, either MS11 or R10, bound only the region corresponding to residues 48-60 (Fig. 3, A and C). As expected for a common, immunorecessive determinant, both sera bind equally well, but significantly less than the sera bind the strain-specific epitopes. Neither sera bound peptides corresponding to residues 21-35, 41-50, or 69-84.

A different binding pattern is evident when antisera against the cyanogen bromide-generated fragment, 8-92, of either MS11 or R10 is used (Fig. 3, B and D). Anti-MS11 CNBr-2 binds the peptide corresponding to residues 69-84 more effectively than 48-60. Anti-R10 CNBr-2 does not bind 48-60 at all. Instead, this serum binds regions corresponding to residues 69-84 and 41-50.

These experiments indicate that an epitope common to both R10 and MS11 pili exists between residues 48-60 and that the immunogenicity of this region is determined by interactions present in intact pili but absent from CNBr-2. Another common determinant, corresponding to residues 69-84, evidently is exposed when CNBr-2 is used as an immunogen.

A solid phase radio-binding assay was performed with the MS11 and R10 anti-whole pili sera to more precisely compare the relative amount of antibodies bound by the common and each of the strain-specific determinants. Table III is a compilation of the results of this experiment. At a 1 to 50 dilution of anti-MS11 pili sera approximately three times as many antibodies are bound by each of the strain-specific epitopes as by the common determinant. Consequently <15% of the antigenicity of whole pili resides in the common determinant, consistent with the data shown in Fig. 1.

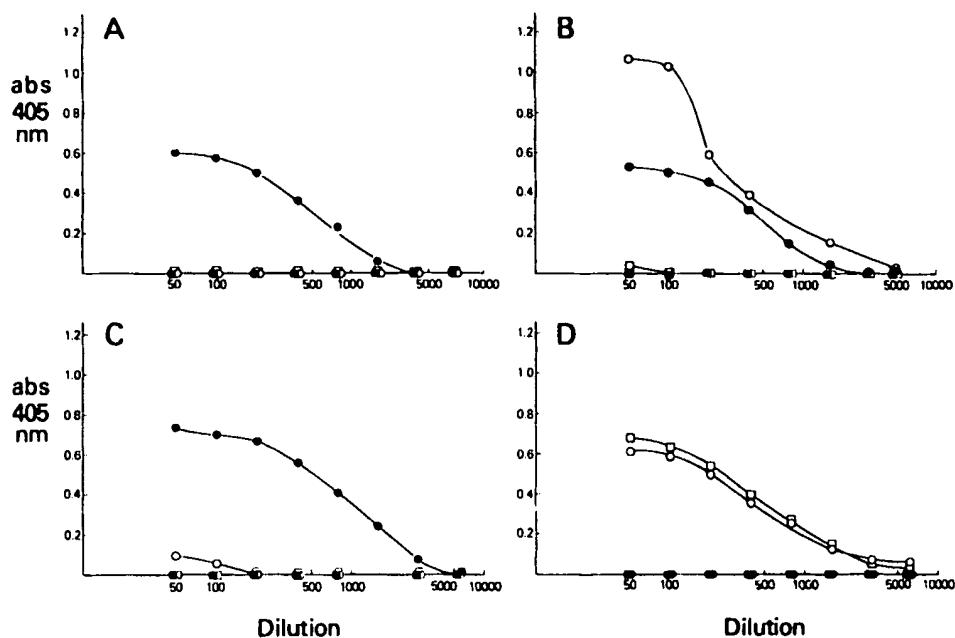


FIGURE 3. Reaction of anti-gonococcal pili sera with peptide-BSA conjugates corresponding to regions of conserved sequence between strains. Anti-MS11 pili (A), anti-MS11 CNBr-2 (B), anti-R10 pili (C), and anti-R10 CNBr-2 (D) antisera were assayed for their ability to bind peptides corresponding to residues 21-35 (■), 41-50 (□), 48-60 (●), and 60-84 (○). The enzyme immuno-binding assays were performed as described in Materials and Methods. Each point represents the average of three determinations.

TABLE III
Relative Binding of Polyclonal Rabbit Sera Against Intact MS11 Pili to Each of the Peptide-BSA Conjugates*

	BSA	21-35	41-50	48-60	69-84	95-107	107-121	121-134	135-151
	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm
Anti-MS11	612 ± 12	656 ± 21	810 ± 28	6,519 ± 112	649 ± 15	635 ± 26	1,824 ± 41	17,251 ± 127	18,020 ± 95
1:50	—	—	—	14.5	—	—	—	40.7	42.6
Relative %	—	—	—	—	—	—	—	—	—

* Wells were coated with 10 µg/ml of the various peptide-BSA conjugates, washed, treated with a 1:50 dilution of anti-MS11 sera, washed, exposed to 50,000 cpm of ¹²⁵I protein A, washed, and the wells were cut from the plate and counted.

Discussion

Defining the antigenic structure of gonococcal pili is important for both the design of an efficacious vaccine and for providing molecular details that will lead to a general understanding of how pathogens create antigenic variation to avoid the host immune system.

The experiments described in this paper have partially defined the natural epitopes of gonococcal pili by using synthetic peptides corresponding to linear sequences of the protein. Two strain-specific determinants are located within the disulfide loop near the carboxyl terminus and a single epitope common to both strains R10 and MS11 is present between residues 48 and 60. We recognize that these areas might not constitute the complete antigenic structure of pili because

if there are any determinants composed of nonlinear amino acids, created by the juxtaposition of residues due to either the folding of the pilin molecule or its polymerization, they would not be detected by these reagents. However, an analysis of the regions containing the linear epitopes indicates that they can account for many of the interesting features of the antigenicity of gonococcal pili.

The location of the three peptides containing the antigenic determinants (48–60, 121–134, and 135–151) as well as the five other areas synthesized within the MS11 pilin sequence are shown in Fig. 4. This figure also includes plots of the protein's hydrophilicity and its predicted secondary structure. Table IV compares the sequences of R10 and MS11 pilin in the eight regions synthesized as well as the quantitative predictions for β -turns using the values compiled by Chou and Fasman (15). All known sequence variation between R10 and MS11 pilin occurs in the carboxyl terminal cyanogen bromide-generated fragment (CNBr-3, residues 93–159) (7). The most striking feature of the variations in sequence between R10 and MS11 is that most of the differences occur within or adjacent to predicted β -turns and yet none of the substitutions affect the predicted secondary structure (Table IV).

The initial choice of the peptides synthesized was based on several criteria, including the predicted location of β -turns. The inherent characteristics of reverse turns makes them attractive both as immunogens within synthetic peptides and as possible antigenic determinants in globular proteins. When a synthetic peptide is used as an immunogen, the possible conformations that it can adopt will determine which clones in the B cell repertoire are stimulated to produce antibody. Only if a peptide adopts a secondary structure identical to

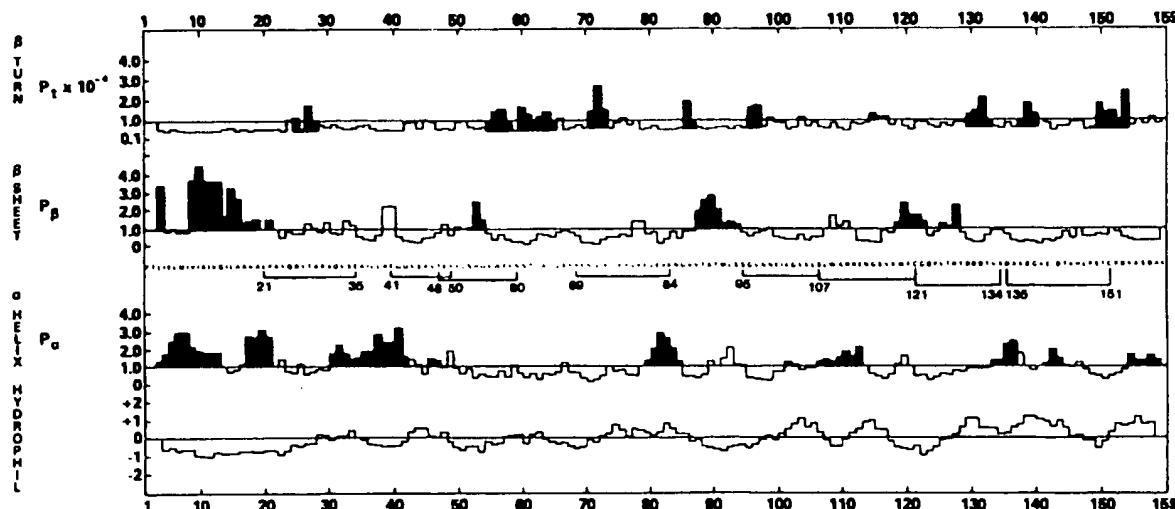


FIGURE 4. Location of the eight oligopeptides within the sequence of MS11 pilin, their predicted secondary structure (15), and their relative hydrophilicity. The relative hydrophilicity of the protein was calculated by a moving average over six residues using the values assigned by Levitt (16). Positive values correspond to the hydrophilic regions. The shaded areas indicate regions of probable conformation.

TABLE IV
Comparison of MS11 and R10 Pilin Sequences in the Regions Synthesized and the Predicted Location of β -Turns

		Sequences											Turn probability*							
MS11	21	L	P	A	<u>Y</u>	Q	D	<u>Y</u>	T	A	R	A	Q	V	S	E	35	1.79×10^{-4}		
R10		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	†	
MS11	41	E	G	<u>Q</u>	K	S	A	V	T	E	Y	50							0.76×10^{-4}	
R10		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MS11	48	T	E	Y	Y	L	N	H	G	K	W	P	E	N	60			1.49×10^{-4}		
R10		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.62×10^{-4}	
MS11	69	P	P	S	D	I	K	G	K	Y	V	K	E	V	E	V	K	84	3.10×10^{-4}	
R10		[]		‡	
MS11	95	S	G	V	N	N	E	I	<u>K</u>	<u>G</u>	<u>K</u>	<u>K</u>	L	S	107			0.89×10^{-4}		
R10		—	N	—	—	K	—	—	D	—	—	—	—	—	—	—	—	—	0.84×10^{-4}	
MS11	107	S	L	W	A	R	R	<u>E</u>	<u>N</u>	<u>G</u>	S	V	K	W	F	C	121	0.94×10^{-4}		
R10		—	—	—	—	K	—	—	A	—	—	—	—	—	—	—	—	—	0.86×10^{-4}	
MS11	121	C	G	Q	P	V	T	R	T	D	D	D	T	V	A	134			2.29×10^{-4}	
R10		—	—	—	—	—	—	—	A	—	K	—	N	R	T	—	—	—	2.75×10^{-4}	
MS11	135	+ D	A	K	D	G	K	E	I	D	T	K	H	L	P	S	T	C	151	1.88×10^{-4}
R10		— V	— D	A	[]		1.81×10^{-4}

* Probability of the underlined four amino acids to be present in a reverse turn based on the algorithm derived by Chou and Fasman (15). Regions whose probability is greater than 0.7×10^{-4} are assigned as turns by this algorithm. Relative charge differences between the two sequences are shown above the indicated position of the MS11 peptide.

† —— indicates sequence identity between MS11 and R10 pilin.

‡ Two overlapping turns are predicted in this region, HGKW and WPEN.

§ [] indicates unsequenced regions of R10 pilin.

that it assumes in the intact protein will it both bind sera generated against the intact protein and elicit antibodies that cross-react with that protein. Even if the antibody-combining site induces the peptide to adopt the correct conformation, a peptide having a higher probability to preexist in that conformation will have the lowest energy requirements for binding. Of the three common secondary structures present in proteins, β -pleated sheets, α -helices, and reverse or β -turns, we believe reverse turns have the highest probability of being present in an isolated oligopeptide < 15 residues in length. Individual strands of a sheet require interactions with the adjacent strands to adopt that conformation and to be subsequently stabilized (17). Helices can form in an isolated peptide, but the peptide must be long enough to generate sufficient intrahelical interactions to stabilize the conformation (18). Further stabilization often results from a particular face of a helix interacting with adjacent regions of the protein (17). In

contrast, the forces involved in the formation of a reverse turn are thought to be contained within the primary structure of the peptide (19). Therefore, a peptide containing a region with high turn probability will be more likely to be identical to the corresponding region in the intact protein than regions predicted to be sheets or helices.

Turns also are present in immunologically interesting regions in proteins. A number of investigators have postulated that turns exist as a means of keeping a protein globular by reversing the direction of a sheet or helix back into the center of a protein, which results in the turn being present on the surface of a protein (20-24). Turns also are likely regions for advantageous mutations to occur within microbial proteins exposed to host immunity. Unlike sheets and helices, the amino acid side chains in turns interact with the solvent and not with other regions of the protein. Consequently, as long as the substitution of an amino acid maintains the turn there is no need to mutate a second amino acid to conserve the correct tertiary structure of the protein, as might be the case if the mutation occurs in a helix or sheet.

The experiments reported in this paper support this proposal. Both of the peptides containing strain-specific determinants (121-134 and 135I-151) have a predicted turn around which the sequence variations are localized (Table IV). Each peptide also contains a turn within which there exists a position where one strain has a lysine, whereas the other contains an aspartic acid. Such a variation in sequence involving a double charge change could explain the total absence of cross-reactivity of the complementary antibody-combining sites. Another feature of the strain-specific determinants is that they are localized within a disulfide loop, a region of the protein previously shown not to be involved in either pilin polymerization or receptor binding (8). Single disulfide loops are also found in *Moraxella bovis* (G. K. Schoolnik, unpublished observations), *Pseudomonas aeruginosa* (25), and common and Gal-Gal binding *Escherichia coli* (26, 27) pili. We believe that the location of the principal epitopes within the disulfide loop is part of a sophisticated molecular design that enables the organism to segregate its principal antigenic determinants in a region distinct from the residues involved in receptor binding and subunit polymerization. By using a disulfide loop, we propose that the molecule has evolved a domain that (a) prominently orients the region on the surface of the molecule, perhaps even projecting out from the polymeric structure, resulting in its increased antigenicity and immunogenicity; (b) allows amino acid substitutions, additions, and deletions within the antigenic determinants, thereby maximizing antigenic variation, without disrupting areas critical for the biological role of the protein; and (c) permits an antibody to bind pili and yet not interfere with gonococcal adhesion to the mucosal cell surface. The highly variable antigenic determinants of influenza virus neuraminidase recently have been shown to be designed in a similar fashion. They are located in β -turns, in a region distinct from the active site, which enables antibodies to bind to the epitopes without inhibiting the enzyme (28).

In addition to making the regions within the disulfide loop immunodominant, the design of pilin also has resulted in the observed regions involved in receptor binding being immunorecessive. Our experiments have shown that the small, cross-reactive antibody fraction of anti-pili sera is directed at an epitope located

between residues 48 and 60. The poor immunogenicity of this region compared with the strain-specific determinants explains why pili from a single gonococcal strain are not suitable immunogens for use as a vaccine. In contrast, the central cyanogen bromide-generated fragment (CNBr-2, residues 8-92) elicits anti-serum that cross-reacts well with heterologous gonococcal pili (8, Fig. 1). Experiments in this paper indicate that the principal epitope of this fragment is not between residues 48 and 60, but rather between residues 69 and 84, an area not recognized by sera against intact pili. The immunogenicity of the region corresponding to residues 69-84 apparently is greatly reduced in the intact protein.

The immunogenicity and functional properties of antibodies engendered by the synthetic peptides described in this report and to the fragments from the conserved, receptor-binding region of the pilin sequence currently are being studied to identify suitable components for a vaccine.

Summary

The antigenic structure of gonococcal pilin, strain MS11 (Tr), was investigated by assaying the binding of antisera engendered by intact pili from strains MS11 and R10 and their two major cyanogen bromide-generated fragments, CNBr-2 (residues 9-92) and CNBr-2 (residues 93-159), to synthetic peptides corresponding to the amino acid sequence of MS11 pilin. Four peptides were synthesized corresponding to regions of sequence variation between MS11 and R10 gonococcal pilin. Antisera against the homologous pilus filament and against its CNBr-3 fragment bind peptides equivalent to residues 121-134 and 135-151, which comprise the 30 amino acid disulfide loop near the carboxyl terminus of the protein. Heterologous pili antisera did not bind these peptides. Absorption studies proved that each peptide contained an independent, strain-specific epitope. Synthetic peptides corresponding to regions of identical sequence between MS11 and R10 pilin were used in similar binding experiments to localize a weakly immunogenic, common determinant between residues 48 and 60. <15% of the antibodies raised against intact pili were directed at this site. Antisera raised against MS11 or R10 CNBr-2 bind a separate peptide, residues 69-80. This region is immunogenic only as a fragment, not in the intact pilus filament.

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